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Canadian Patent

Brevet canadien

1270781

To all to whom these presents shall come:

Whereas a petition has been presented to the Commissioner of Patents praying for the grant of a patent for a new and useful invention, the title and description of which are contained in the specification of which a copy is hereunto attached and made an essential part hereof, and the requirements of the Patent Act having been complied with,

Now therefore the present patent grants to the applicant whose title thereto appears from the records of the Patent Office and as indicated in the said copy of the specification attached hereto, and to the legal representatives of said applicant for a period of seventeen years from the date of these presents the exclusive right, privilege and liberty of making, constructing, using and vending to others in Canada the invention, subject to adjudication in respect thereof before any court of competent jurisdiction.

Provided that the grant hereby made is subject to the conditions contained in the Act aforesaid.

In testimony whereof, these letters patent bear the signature of the Commissioner and the seal of the Patent Office hereunto affixed at Hull, Canada.

A tous ceux qui les présentes verront:

Considérant qu'une requête a été présentée au Commissaire des brevets, demandant la délivrance d'un brevet pour une invention nouvelle et utile, dont le titre et la description apparaissent dans le mémoire discriptif dont copie est annexée aux présentes et en fait partie essentielle, et que ladite requête satisfait aux exigences de la Loi sur les brevets,

A ces causes, le présent brevet confère au demandeur dont le titre de propriété audit brevet est établi d'après les dossiers du Bureau des brevets et est indiqué dans ladite copie du mémoire descriptif ci-annexé, et aux représentants légaux du dit demandeur, pour une période de dix-sept ans, à compter de la date des présentes, le droit, la faculté et le privilège exclusif de fabriquer, construire, exploiter et vendre à d'autres au Canada l'invention, sauf jugement en l'espèce par un tribunal de juridiction compétente.

La concession faite par les présentes étant soumise aux conditions contenues dans la loi précitée.

En foi de quoi ces lettres patentes portent la signature du Commissaire ainsi que le sceau du Bureau des brevets apposé à Hull, Canada.

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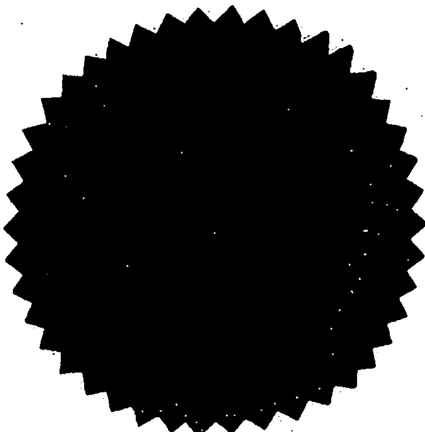

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(19) (CA) CANADIAN PATENT (12)

(54) Method for Production of an Immobilized Lipase Preparation and Use Thereof

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Immobilized lipase preparations for transesterification of fats are known. Thus, in U.S. Patent No. 4,275,081, Coleman et al issued June 23, 1981, an immobilized lipase preparation is described, whereby the lipase is produced by fermentation of species belonging to the genera Rhizopus, Geotrichum or Aspergillus, and whereby the lipase is attached on an indifferent particulate carrier which may be diatomaceous earth or alumina, and which exhibits a very high specific surface area. It has been deemed necessary to use an immobilized lipase preparation with very high specific surface area (i.e. small and porous carrier particles) in order to obtain the necessary high enzymatic activity. Interesterification can be carried out batchwise without a solvent with this immobilized lipase preparation; however, with this immobilized lipase preparation continuous interesterification in a column cannot be carried out on an industrial scale without the presence of a solvent, which has to be removed later, due to the above indicated fact that the preparation consists of small particles, which during column operation would generate an unacceptably high pressure drop. Also, a poster presented at Enz.Eng. 6, Kashikojima, Japan, 20 - 25 Sept. 1981 and the article in European Journal of Applied Microbiology and

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Biotechnology, No. 14, pages 1 - 5 (1982) indicates that an immobilized lipase preparation comprising lipase from Rhizopus delemar and a strong anion exchange resin (with quaternary amino groups) may be used for interesterification with n-hexane as a solvent. The enzyme recovery according to these references is very low, though. Also, from European patent application published with publication No. 0069599 an enzymatic rearrangement of fat is described, whereby a lipase from Aspergillus species, Rhizopus species, Mucor javanicus, or Mucor miehei is used as an interesterification enzyme. The enzyme is supported on a carrier, e.g. Celite. In all examples in this European patent application relating to continuous interesterification in a column a solvent is used.

Thus, in prior art processes the solvent is used to lower the viscosity of the fatty starting material in order to secure as smooth a column operation as possible. It has hitherto been deemed practically impossible to avoid solvent in these continuous interesterification processes on an industrial scale, due to the high pressure drop in the column, even if the technical advantages associated with the elimination of solvent from these interesterification processes are quite obvious.

In European patent application published as no. 35.883 it is described that an immobilized lipase preparation intented for interesterification of fats can be prepared by contacting an aqueous solution of microbial lipase with a particulate, inert carrier followed by drying. The thus prepared immobilized lipase preparation can be used for interesterification of fats, but only if the fats are mixed with the relatively expensive low alkyl esters of fatty acids, e.g. methyl palmitate, which are used as auxiliary agents in order to avoid solvents; otherwise solubility and viscosity problems arise.

Thus, the purpose of the invention is to provide a method for production of an immobilized lipase preparation and to provide a preparation per se which will open up the possibility of carrying out the ----------------------

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continuous interesterification without a solvent or other expensive auxiliary agents in an economically feasible way.

Now, surprisingly according to the invention it has been found that a method for production of an immobilized lipase preparation, which is very easily performed, viz. by simple mixing of an aqueous solution of lipase and an ion exchange resin and which comprises a specific combination of a specified category of ion exchange resins and a specified proportion of water in the final immobilized lipase preparation opens up the possibility of carrying out the continuous interesterification without a solvent or other expensive auxiliary agents in an economically feasible way.

Thus, the method according to the invention for production of an immobilized lipase preparation intended for interesterification of fats is characterized by the fact that an aqueous solution of a microbial lipase is contacted with a particulate, macroporous, weak anion exchange resin which contains primary and/or secondary and/or tertiary amino groups and which exhibits a relatively large average particle size suitable for column operation without excessive pressure drop, at conditions, at which the lipase is bonded to the anion exchange resin during a period of time sufficient to bind the wanted amount of lipase to the anion exchange resin, whereafter the thus formed immobilized lipase is separated from the aqueous phase and the separated immobilized lipase is dried to a water content of between approximately 2 and 40%.

It is described generally in German published patent applicatons nos. 2 905 671 and 2 805 950, Japanese published patent applications nos. 54-76892 and 57-152886, US patent no. 4 170 696 and Chem.Abs. Vol 82, 27819d that enzymes, including lipases can be immobilized by means of particulate anion exchange resins. In the first place, however, no universal immobilization method suited for all enzymes and all substrates exists, but a specific immobilization method has to be devised for any specific enzyme and any specific substrate, on which the enzyme is

supposed to act. In the second place, however, lipases are quite extraordinary enzymes in the sense that the enzymatic activity is functioning on an interphase between two phases, meaning that the immobilization of the lipases is a very delicate problem, which highly limits the utility of known immobilization techniques in the field comprising lipase immobilization, vide J. Lavayre et al., Preparation and Properties of Immobilized Lipases, Biotechnology and Bioengineering, vol. XXIV, pp 1007 - 1013 (1982), John Wiley & Sons. In the third place the combination of lipase and particulate macroporous weak anion exchange resin is not described in any of the literature indicated in the beginning of this paragraph, and even less that this new combination gives rise to the surprising technical effect in regard to continuous interesterification without solvent or other expensive auxiliary agents.

Also, from an article of Yoshiharu Kimura et al., "Application of Immobilized Lipase to Hydrolysis of Triacylglyceride" in Eur.J.Appl.Microbiol.Biotechnol. (1983) 17:107-112 it appears that a lipase preparation immobilized on an anion exchange resin has been used for hydrolysis of fats. In the first place the main application of the immobilized lipase preparation produced by means of the method according to the invention is interesterification, whereas hydrolysis of fats and synthesis of fats are less important applications according to the invention, which will be explained more detailed later in this specification, and in the second place it appears from the article that the activity yield is less than 1%, vide table 1 on page 109, as compared to an activity yield typically above 80% in relation to the immobilized lipase preparation prepared by means of the method according to the invention. This confirms our previous statement that immobilization of lipase is a very delicate problem.

It is intended that the expression "relatively large average particle size" is related to the average particle size of the product which is described in U.S.

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Patent No. 4,275,081 and of which the majority of the particles have a particle size less than approximately 50 µm. It has been found that the temperature has no great effect on the activity yield, as it has been shown experimentally that the activity yield is virtually temperature independent in case the temperature during immobilization is kept between 5 and 35°C.

In order not to inactivate the enzyme the prior art interesterifications are carried out at relatively low temperature. This is made possible by the presence of the solvent, which is able to dissolve the fat, which might have a relatively high melting point. Surprisingly it has been found that the immobilized lipase preparation produced by means of the method according to the invention has a sufficient stability in the melted fat with a relatively higher temperature. Also, the pressure drop through the interesterification column loaded with the immobilized lipase preparation produced by means of the method according to the invention is sufficiently low to allow a smooth operation. Also, surprisingly it has been found that the unique combination of contact conditions, ion exchange resin and water content generates a high specific lipase activity in the melted fat mixture in contradistinction to all previous attempts to provide an immobilized lipase preparation intended for use without a solvent. Furthermore, whereas prior art processes of the kind described in U.S. Patent No. 4,275,081 have required a purified lipase in order to provide a usable immobilized preparation, it has surprisingly been found that the immobilized lipase preparation according to the invention can be prepared on the basis of a rather crude lipase product. Also, whereas the preparation of prior art processes of the kind described in U.S. Patent No. 4,275,081 have involved the use of an organic solvent to deposit the lipase on the carrier, no such organic solvent is needed for the preparation of the immobilized lipase preparation according to the invention, which can be prepared very easily just by mixing of carrier and an aqueous lipase solution. Furthermore, whereas the lipase

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activity is relatively easily washed off or otherwise removed from the prior art preparation of the kind described in U.S. Patent No. 4,275,081, it has been found that the lipase in the immobilized lipase preparation produced by means of the method according to the invention is practically impossible to remove from the preparation, if not subjected to adverse chemical or physical treatments, for instance adverse pH and temperature conditions. Finally it has been found that the immobilized lipase preparation produced by means of the method according to the invention can be prepared with a high enzyme recovery which opens up the possibility for a cheaper continuous interesterification than the prior art interesterifications.

In a preferred embodiment of the present invention the lipase is a thermostable lipase. Hereby a higher interesterification temperature is made possible, and thus a higher productivity. Furthermore by means of this embodiment it is possible to produce an immobilized lipase preparation which is well suited for interesterification of higher melting fats.

In a preferred embodiment of the present invention the microbial lipase is derived from a thermophilic Mucor species, especially Mucor miehei. Mucor miehei is a good producer of 1,3-specific lipase, and thus a cheap product can be obtained.

In a preferred embodiment of the present invention more than 90% of the particles of the macroporous weak anion exchange resin have a particle size between approximately 100 and 1000μm, preferably between 200 and 400 μm. In this particle size interval a good compromise between high interesterification activity and low pressure drop is obtained.

In a preferred embodiment of the present invention the proportion between the amount of the aqueous solution of the microbial lipase and the weight of weak anion exchange resin corresponds to 5,000 - 50,000 LU/g ion exchange resin (dry weight). In this manner sufficient lipase for the ion exchange resin is provided.

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In a preferred embodiment of the method according to the invention the microbial lipase is derived from a thermophilic Mucor species, especially Mucor miehei, and the pH during contact between ion exchange resin and aqueous solution is between 5 and 7. In this manner a strong bond between lipase and ion exchange resin as well as a good stability and activity is secured.

In a preferred embodiment of the method according to the invention the contact time is between 0.5 and 8 hours. In this manner a state of saturation with lipase is obtained or approximated.

In a preferred embodiment of the method according to the invention the separation is performed by simple filtration. This process is simple and well adaptable to industrial practise.

In a preferred embodiment of the method according to the invention the drying is carried out to a water content between approximately 5 and 20% of water. The drying operation can be carried out in vacuum, in fluid bed or by other drying means suitable for large scale operation. Hereby a final lipase preparation with a high interesterification activity is obtained.

In a preferred embodiment of the method according to the invention the particulate, macroporous weak anion exchange resin is brought together with an aqueous solution of a crosslinking agent, preferably an aqueous glutaraldehyde solution in a concentration between 0.1 and 1.0% w/w, before, during or after the contact between the particulate, macroporous, weak anion exchange resin and the aqueous solution of the microbial lipase, whereafter the remaining solution of crosslinking agent is separated. Even if a minor reduction of enzyme activity may be observed due to the crosslinking agent it has been found that such treatment may raise the stability of the lipase preparation in aqueous media for a specific application. In relation to use of the immobilized lipase preparation as an interesterification agent there is no need for any improvement of the stability of the lipase preparation, as

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this stability is inherently excellent in the lipase preparation prepared according to the invention for this application. However, it has been found that the immobilized lipase preparation prepared in accordance with the method according to the invention advantageously can be used for hydrolysis of fats as well, and for this application an improvement of the stability of the lipase preparation is a desideratum, probably due to the combination of relatively high concentration of water and high temperatures in the reaction mixture, necessary for an industrially performed fat hydrolysis process.

Also, the invention comprises the immobilized lipase preparation so formed and use of the immobilized lipase preparation according to the present invention for interesterification of fats, wherein melted fat(s), facultatively mixed with dissolved free fatty acid, is contacted with the immobilized lipase prepared by means of the method according to the invention, without any solvent or other expensive auxiliary agents or substantially without any solvent or other expensive auxiliary agents. The free fatty acids, with which the fats facultatively may be mixed according to the invention are not to be considered as expensive auxiliary agents. By fat(s) is meant either a pure triglyceride or a mixture of triglycerides from one or more sources.

Also, the invention comprises another use of the immobilized lipase preparation prepared by means of the method according to the invention, which is a method for hydrolysis of fats, wherein an emulsion of triglyceride and water is contacted with the immobilized lipase preparation produced according to the invention without any solvent or other expensive auxiliary agents or substantially without any solvents or other expensive auxiliary agents.

Also, the invention comprises another use of the immobilized lipase preparation prepared by means of the method according to the invention, which is a method for synthesis of fats wherein a mixture of glycerol and free fatty acids is contacted with the immobilized lipase preparation produced according to the invention without any

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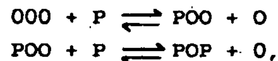
solvent or other expensive auxiliary agents or substantially without any solvents or other expensive auxiliary agents.

The invention will be illustrated by the following examples.

The Mucor miehei lipase used in the following examples can be obtained from NOVO Industri A/S, Novo Alle, 2880 Bagsvaerd, Denmark, on request (as enzyme product SP 225). This Mucor miehei lipase can be obtained as indicated in Danish published patent application No. 145,285, published February 28, 1983.

The lipase activity unit (LU) indicated in the examples is determined as described in the publication AF 95.1/2-GB of 83-01-03, obtainable from NOVO Industri A/S, Novo Alle, 2880 Bagsvaerd, Denmark.

The interesterification activity of the immobilized lipase preparations is determined by means of a batch assay based on the following reactions:



$$\text{OOO} + \text{P} \rightleftharpoons \text{POO} + \text{O}$$
$$\text{POO} + \text{P} \rightleftharpoons \text{POP} + \text{O},$$

where O = oleic acid, P = palmitic acid, and OOO, POO and POP are fats containing the indicated fatty acids in the order indicated, OOO thus being triolein.

250 mg of immobilized lipase preparation is mixed with 600 mg triolein (0.68 mmol) and 174 mg palmitic acid (0.68 mmol) dissolved in 12 ml petroleum spirit (temp. 80 - 100°C) in a 20 ml glass tube with screw cap. The tubes are incubated in a water bath at 40°C and shaked for 1/2, 1 or 3 hours.

The reaction mixture is cooled, filtered and evaporated. The relative amount of OOO, POO and POP is determined by HPLC, and the percentage of incorporated P is calculated as

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$$\% \text{ incorporated P} = \frac{\% \text{ POO} + 2 \times \% \text{ POP}}{3}$$

The equilibrium composition of the above indicated batch reaction mixture is approximately 43% POO and 10% POP or 21% incorporated P.

In some of the following examples the interesterificaton is carried out as a batch operation with or without solvent. By comparative tests it has been established that an immobilized lipase preparation, which has satisfactory interesterification activity and stability, as demonstrated by the batch interesterification test, and which possesses a particle size distribution and a physical strength required for satisfactory column operation, will operate satisfactorily by continuous operation in column as well, with or without solvent. Thus, a satisfactory batch test under these circumstances is evidence that a satisfactory continuous column test can be carried out with the immobilized lipase preparation in question.

Figure 1 and Figure 2 are plots of the logarithm to the flow rate (of the substrate per hour) versus time at 60°C and 70°C,respectively.

Example 1

This example illustrates the effect of pH during adsorption of Mucor miehei lipase on interesterification activity.

2.0 grams of Mucor miehei lipase, 93,000 LU/g, was dissolved in 20 ml of water, and 10 grams of water washed Duolite ES 562 anion exchange resin, dry weight 8.5 g, was suspended herein.

Three such portions were adjusted to pH 5.0, 6.0, and 7.0 respectively and left agitated with magnetic stirring for 4 hours at approximately 5°C.

The three portions were filtered. After filtration the amount of hydrolytic activity (LU) in the three filtrates (before wash) was between 10 and 17% of the total, initial amounts (186,000 LU). Subsequently a water wash was carried out with a small amount of water, and thereafter the preparations were dried overnight in vacuum at room temperature.

The results are summarized in the table below.

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Immobilization pH	Yield g	Water content %	Interesterification activity, 30 minutes % POO	% POP	% incorporated P
5.0	9.20	9.5	24.5	6.2	12.3
6.0	9.56	8.2	26.5	6.6	13.2
7.0	9.41	8.0	21.2	5.2	10.5

Example 2

Three 10 g portions of moist ion exchange resin, Duolite ES 562* (dry weight 8.35 g) were suspended in 50 ml of water, and 4 N NaOH was added until pH stabilized at 6.0. Then they were washed with plenty of water and drained on a Büchner funnel, drained weight approximately 16 g.

To each one of two 10 g portions was added a solution of 2.5 g Mucor miehei lipase (activity 93,000 LU/g) in 25 ml of water, and pH was adjusted to 6.0.

To the third portion was added a solution of 2.5 g of the above indicated Mucor miehei lipase in 50 ml of water, and pH was adjusted to 6.0.

The mixture was slowly agitated at room temperature (25°) for 2 hours. Hereafter the liquid was filtered off on a Buchner funnel.

One of the portions with 25 ml lipase solution was furthermore washed with 2 x 25 ml of water. The immobilized preparations were dried in vacuum.

For the interesterification assay 250 mg (dry weight) of the immobilized lipase preparations were moistened with 20 µl of water prior to mixing with the substrate.

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Lipase preparation immobilized with	Interesterification, 1/2 hour		
	%POO	%POP	% incor-porated P
2.5 g lipase in 25 ml without wash	25.8	6.85	13.2
2.5 g lipase in 25 ml with wash	30.1	7.65	15.1
2.5 g lipase in 50 ml without wash	26.8	6.86	13.5

This example demonstrates that subsequent water wash in order to remove unbound lipase is essential for obtaining a high interesterification activity, whereas the amount of water in which the lipase is dissolved during immobilization, is of minor importance.

Example 3

50 g of moist ion exchange resin Duolite ES 562* (dry weight 41.8 g) was adjusted to pH 6.0 and washed as in example 2.

10.6 g portions of this moist ion exchange resin (~5 g dry weight) were mixed with different amounts of a 10% solution of Mucor miehei lipase (81,000 LU/g) according to the table.

After the reaction the liquid was filtered off on a Buchner funnel, and the lipase preparation was washed with 2 x 25 ml water and dried in vacuum to approximately 97% dry matter.

The 250 mg dry weight samples of immobilized preparation for assay purposes were moistened with 20 µl water prior to assaying.

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g of moist ion exchange resin	g of 10% solution of lipase	Reaction time, hours at room temperature	Interesterification, 1/2 hour % POO	% POP	% incorporated P
10.6	12.5	1	26.5	6.62	13.3
10.6	12.5	2	27.0	6.62	13.4
10.6	12.5	4	28.2	7.27	14.3
10.6	25	1	23.5	5.90	11.8
10.6	25	2	29.7	7.56	14.9
10.6	25	4	31.4	7.99	15.8
10.6	50	1	19.5	4.34	9.4
10.6	50	4	26.6	6.73	13.4

This example shows that the optimal dosage of lipase depends upon the reaction time.

Example 4

Two of the preparations from example 3 were reassayed with varying addition of water, i.e. the sample with 12.5 g lipase solution and that with 25 g lipase solution, both with a reaction time of 2 hours. The effect of the moisture content on the interesterification activity appears from the following table.

Sample	µl water added to 250 mg dry weight	Estimated moisture in sample %	Interesterification, 1/2 hour % POO	% POP	% incorporated P
12.5 g	0	2.6	18.2	2.27	7.6
	20	9.6	25.6	6.55	12.9
	50	18.5	23.4	5.85	11.7
	100	29.9	15.3	3.84	7.6
25 g	0	3.0	19.1	2.04	7.7
	20	10.0	28.6	7.65	14.6
	50	18.8	25.4	5.25	12.0
	100	30.1	18.6	4.55	9.2

This example shows that the optimal moisture content is approximately 10%.

Example 5

One of the preparations from example 3 was reassayed with varying amounts of added water. The sample

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with 25 g lipase solution and 4 hours reaction time was used.

µl water added to 233 mg dry weight	Estimated water in sample, %	Interesterification, 1/2 hour		
		% POO	% POP	% incorporated P
0	9.5	28.0	6.57	13.7
10	13.1	28.9	7.45	14.6
20	16.2	27.9	6.46	13.6
30	19.1	26.6	6.96	13.5
40	21.8	25.0	6.77	12.8
50	24.4	22.8	5.20	11.1
75	30.0	19.6	4.54	9.6
100	34.9	14.6	3.88	7.5
150	42.9	0.44	0	0.1

Example 6

22.8 g of moist ion exchange resin Duolite A 561* (88.2% dry substance) was adjusted to pH 6.0 and washed.

Another 22.8 g sample of Duolite A 561* was crushed partially in a mortar prior to pH adjustment and washing.

To each of these portions were added a solution of 10 g Mucor miehei lipase (93,000 LU/g) in 200 g of water adjusted to pH 6. Reaction took place in 2 hours at room temperature.

The immobilized enzymes were washed with 1 liter of water and dried in vacuum.

After drying the uncrushed sample was crushed in a mortar, and both samples sieved.

Sieve fraction	Interestification, 1/2 hour Crushing prior to immobilization			Crushing after immobilization		
	% POO	% POP	% incorp. P	% POO	% POP	% incorp. P
180 - 300 µm	30.1	7.78	15.2	25.7	6.39	12.8
425 - 500 µm	25.7	6.66	13.0	21.7	5.50	10.9
600 - 710 µm	19.2	5.06	9.8	17.2	4.38	8.7
850 - 1000µm	12.7	3.22	6.4	14.3	3.90	7.4

It clearly appears that it is an advantage to use the fine sieve fractions to obtain maximum interes-

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terification activity, but the need for a low column pressure drop makes a compromise necessary.

Example 7

This example illustrates the effect of different categories of macroporous weak anion exchange resins (type of matrix, functional groups, particle size) on the batch interesterification activity of the immobilized lipase preparation.

In the case of Duolite ES 562*, Duolite A 561*, Duolite A 7*, Amberlite IRA 93*, and Amberlyst A 21* 4.25 grams dry weight resin was washed with water, mixed with 1 gram of Mucor miehei lipase (93,000 LU/g) in 20 ml of water, the mixture being adjusted to pH 6.0, and rotated slowly for 2 hours at room temperature. After filtration, each preparation was washed with 250 ml of water. In the case of Duolite A 378* 8.5 grams was mixed with 2 grams of lipase and finally washed with 250 ml of water. All were dried in vacuum at room temperature. In the case of Duolite A 365*, Duolite S 587*, and Dowex MWA-1* 4.25 gram dry weight resin was mixed with 1 gram of Mucor miehei lipase (124,000 LU/g) in approximately 10 ml of water for 2 hours by rotation at room temperature (in the case of Lewatit*, 0.5 of lipase was used, though). After filtration and washing with 2 volumes of water, the preparations were dried in vacuum at room temperature. Characterization of the immobilized preparations is shown in the table below.

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Anion exchange resin	Matrix	Funct. groups	Partc. sizes, μm (>85%)	Water %	Batch % POO	activity, % POP %	1/2 hour incorp. P
Duolite ES 562	Phenol-formalde-hyde	Tert. amine	212 - 425	13.8	26.7	6.8	13.4
Duolite A 561	Phenol-formalde-hyde	Tert. amine	300 - 1200	13.0	14.8	3.2	7.1
Duolite A 7	Phenol-formalde-hyde	Second. amine	300 - 1200	13.5	9.5	2.5	4.8
Duolite A 378	Polysty-renic	Tert. amine	300 - 1100	6.3*	14.3	3.3	7.0
Amberlite IRA 93	Styrene-DVB	Poly-amine	400 - 500	12.2	10.8	2.9	5.5
Amberlyst A 21	Styrene-DVB	Tert. amine	425 - 850	11.1	10.6	2.7	5.3
Duolite A 365	Polysty-renic	Prim. amine	300 - 1200	11.5	15.5	3.7	7.6
Duolite S 587	Phenol-form.	Amines	300 - 1100	7.4	25.4	6.4	12.7
Lewatit MP 62	Polysty-renic	Amines	300 - 1200	13.6	16.9	3.9	8.2
DOWEX MWA-1	Styrene-DVB	Tert. amine	300 - 1200	10.5	21.0	4.9	10.3

* 5% water was added before batch assay

Example 8

30 g Duolite* ion exchange resin type ES 562 was suspended in approximately 75 ml of H_2O, and pH was adjusted to 6.0 with 4 N NaOH. The ion exchange resin was washed with water on a suction filter, and excess of water was sucked away. The wet ion exchange resin (approximately 45 g) was divided in three equal portions.

The first portion was mixed with a solution of 1 g Mucor miehei lipase (210,000 LU/g) in 20 ml of H_2O adjusted to pH 6.0. After mixing the pH was readjusted to 6.0, and the mixture was allowed to react for 4 hours at 5°C with magnetic stirring. During this period the pH dropped to 5.45. The mixture was transformed to a Buchner funnel with a few milliliters of water and as much as possible of the solution was sucked away (14 ml). The resin was further dried in vacuum to a water content of 10.0%. Yield 8.27 g.

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The second portion of the wet resin was mixed with a solution of 1 g of the previously indicated lipase in 20 ml of 0.1 M sodium acetate buffer (pH 6.0). The pH of the mixture was readjusted to 6 and the mixture was allowed to react for 4 hours at 5°C with magnetic stirring. During this period the pH dropped to 5.83. The further procedure was carried out as indicated in relation to the first third of the wet ion exchange resin, giving rise to 21 ml filtrate and 9.10 g dried preparation with a moisture content of 9.5%.

The third portion of the resin was mixed with lipase solution as before, but pH was kept constant at 6.0 during the 4 hour coupling period at 5°C by addition of 0.58 ml 1 N NaOH. The mixture was worked up as the other thirds, giving rise to 28 ml filtrate and 8.95 g dried preparation with 8.9% moisture. The three filtrates contained between 1 and 5% of the initital, total activity.

The interesterification activity with 250 mg immobilized lipase preparation after a reaction time of 30 minutes at 40°C is indicated in the following table.

Enzyme preparation immobilized in	Interesterification activity, 1/2 hour % POO	% POP	% incorporated P
demineralized water, pH 6	27.4	6.6	13.5
0.1 M acetate, pH 6	25.4	6.5	12.8
demineralized water, pH-stat at pH 6	27.7	7.0	13.9

As appears from the table there are only slight differences between the preparations.

Example 9

This example illustrates the effects of the presence of two salts in the concentration range 0 - 0.5 M during immobilization on the interesterification activity.

Five 1.00 gram portions of Mucor miehei lipase, diafiltrated, and freeze-dried, with an activity of 93,000 LU/g, were dissolved in 20 ml of:

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1) demineralized water
2) 0.05 M sodium phosphate, pH 6.0
3) 0.5 M - - , pH 6.0
4) 0.05 M - chloride
5) 0.5 M - -

Other five 5.25 gram portions (dry weight 4.25 g) of Duolite ES 562* ion exchange resin were equilibrated with 20 ml of 1) - 5) above. After decantation, the corresponding lipase solutions were added to the wet ion exchange resin particles adjusted to pH 6.0, and the containers were rotated slowly over 2 hours at 25°C. The preparations were then collected by filtration and each washed with 250 ml demineralized water followed by drying in vacuum at 25°C (64 hours). The results of the interesterification activity assay are shown below:

Salt/concentration	Yield (g)	% H_2O *	Interesterification activity, 1/2 hour % POO	% POP	% incorporated P
No salt	4.51	4.7	23.1	5.7	11.5
0.05 M phosphate	4.48	5.3	21.9	5.3	10.8
0.5 M -	4.57	4.6	20.3	5.1	10.2
0.05 M NaCl	4.54	4.6	23.4	5.7	11.6
0.5 M -	4.43	4.9	19.2	4.6	9.5

* additional H_2O up to a total of 10% was added before assay.

Example 10

This example shows the effects of high concentrations of sodium acetate during lipase immobilization on the interesterification activity of the preparations.

Five 1.00 g portions of Mucor miehei lipase, diafiltrated and freeze-dried, 93,000 LU/g, were separately dissolved in 20 ml of the following liquids:

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1) demineralized water
2) 0.5 M sodium acetate, pH 6.0
3) 1.0 M - - , pH 6.0
4) 2.0 M - - , pH 6.0
5) 4.0 M - - , pH 6.0

Five 4.25 g (dry weight) portions of Duolite ES 562*ion exchange resin were washed and equilibrated by mixing with the five above indicated solutions 1) - 5) followed by shaking for 15 minutes. Corresponding lipase solutions and washed ion exchange resins were mixed, adjusted to pH 6.0 and rotated slowly for 2 hours at room temperature. Each preparation was filtered, washed with 250 ml of water and dried in vacuum at room temperature. The preparations were assayed for batch interesterification activities, the results being shown in the following table.

Acetate conc. (M)	Yield after drying (g)	Water after drying (%)	Filtrate pH	Filtrate Act. (%) *	Batch interesterification activity, 1/2 hour % POO	% POP	% incorporated P
0	4.81	7.8	5.2	51	22.2	5.7	11.2
0.5	4.67	8.0	5.8	64	20.1	4.7	9.8
1.0	4.72	9.6	5.8	71	18.8	4.3	9.1
2.0	4.73	9.1	5.8	55	27.9	7.3	14.2
4.0	4.75	10.4	5.6	69	19.8	4.7	9.7

*) Activity in per cent of total, initial amount (93,000 LU).

Example 11

This example illustrates the immobilization of other microbial lipases than Mucor miehei lipase:

Fusarium oxysporum lipase, prepared as described in ~~Danish~~ Canadian patent application No. ~~3999/84~~, Example 23, was immobilized by mixing 6.72 g of lipase of 88,000 LU/g and 4.25 g dry matter of Duolite ES 562* ion exchange resin, washed and pH-adjusted, in 25 ml water at pH 6.0, and by rotation at room temperature for 2 hours. Then washing was performed with 2 x 25 ml of water, and by vacuum drying 4.93 g of preparation with a water content of 8.1% was obtained.

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The activity left in the total filtrate corresponded to 18% of the original activity.

Aspergillus niger esterase was obtained by ultrafiltration of the commercial product Palatase from NOVO. 15 ml PALATASE of 2790 LU/ml was immobilized on 4.25 g of ES 562* as described above whereby 4.77 g immobilized preparation with 7.6% water was obtained. The filtrate contained 13% of the original LU-activity.

Candida cylindracea lipase from Amano (type OF) was similarly immobilized by mixing 4.25 g of ES 562* with 1.40 g Amano lipase OF in 15 ml of water, pH 6.0. The yield was 4.62 immobilized preparation with 6.5% of water and 0.2% activity remaining in the filtrate.

The three preparations were characterized as follows:

1) By the standard batch assay at 40°C
2) By a triolein (OOO)/decanoic acid (D) batch interesterification without solvent at 60°C using 3.00 g OOO, 0.600 g D, and 250 mg dry lipase preparation hydrated to approximately 10% water.

For comparison purposes also results for a Mucor miehei lipase preparation, as described in example 13, are listed as well:

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Immobilized lipase	Estim. load LU/mg	000/P/solvent, 40° Time (h)	%P inc.	000/D, 60° Time (h)	%D inc.
Fusarium oxysporum	11	3	8.0	17	5.9
Aspergillus niger	8	3	4.4	17	6.5
Candida cylindracea	30	3	8.9	17	1.9
Mucor miehei	30	0.5	14.7	2	13.2

In order to generate a better survey in relation to the preceding examples, reference is made to the following table.

Example No.	This (These) example(s) illustrate(s) the influence on the interesterification activity of the immobilized lipase preparations prepared by means of the method according to the invention originating from
1, 8	pH
2	subsequent wash
3	lipase loading in relation to reaction time
4 - 5	percentage of water
6	particle size
7	type of resin
8 - 10	ion strength in lipase solution
11	microorganism source of lipase

In order to demonstrate the utility of the immobilized lipase preparation prepared by means of the method according to the invention an immobilized lipase preparation prepared by means of the method according to the invention, as indicated later, was used in a continuous interesterification of fats without any use of solvent or

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other expensive auxiliary agents, as described in the following example 12.

Example 12

This example illustrates continuous interesterification of fats without solvent or other expensive auxiliary agents, using an immobilized lipase preparation prepared by means of the method according to the invention in a packed bed reactor.

Immobilization

2.20 grams of Mucor miehei lipase (81,000 LU/g) was dissolved in 20 ml of water, mixed with 10 grams washed (8.5 g dry weight) Duolite ES 562* ion exchange resin with more than 80% of the particles between 200 and 400 µm. The mixture was adjusted to pH 5.0, and left for 4 hours at 5°C with magnetic stirring. After filtration and wash with a small amount of water the preparation was dried in vacuum at room temperature. The yield was 9.05 grams, containing 9.3% water. The activity remaining in the filtrate was 8% of the total, initial amount. The batch interesterification activity was 30.6% POO, 7.7% POP at 1/2 hour or 15.3% incorporated P.

Test in column

2 grams of this immobilized lipase preparation was placed in a column and a solvent-free substrate consisting of olive oil/palmitic acid in the ratio 2.5:1 w/w was continuously fed through at 60°C. The performance of the lipase preparation is shown in the table below.

Sample/time	Flow gTG/h/ g enz.	Composition (HPLC) OOO %	POO %	POP %	Conversion x, % (GLC)
Olive oil/ start	-	42.3	22.5	3.8	0
17 hours	5.7	30.5	30.1	11.6	-
208 1/2 hours	2.5	33.8	28.8	8.6	28
233 -	0.61	22.2	34.8	16.5	67
475 -	1.8	35.1	28.8	8.7	28
Equilibrium (batch)	-	17.4	36.0	20.6	100

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Legend: TG: Triglycerides; g enz. = grams of immobilized lipase
% incorporated P is determined by GLC of fatty acid methyl esters
Conversion x = $(\% P - \% P_0)/(\% P_{eq} - \% P_0)$. P_0, P_{eq} are % incorporated P in the olive oil substrate (P_0) and in the TG-mixture at equilibrium (P_{eq}).

Comments

Based on the 208 1/2 and 475 hours data, extrapolation to start in semilogarithmic plot indicates an initial activity (flow) of 3.2 g TG/h/g enzyme with a corresponding degree of conversion x = 28%. An estimate of the half-life is 500 - 600 hours at 60°C without solvent and olive oil/P = 2 1/2:1 (w/w). No pressure drop problems have been experienced. An earlier attempt to pass a similar substrate through Celite-adsorbed lipase of the kind described in Danish patent application No. 563/77 in a column was impossible.

Example 13

This example illustrates a pilot plant scale production of an immobilized lipase preparation in a column and the application of this preparation for continuous interesterification in a column with substrate at 60 and 70°C, without solvent.

Immobilization

6.0 kg (81% dry matter) Duolite ES 562* ion exchange resin was conditioned according to the manufacturer's information (Duolite Technical Information 0110A). This implies acid-base cycling and in this case also an ethanol rinse (to ensure maximum purity in food processing) pH was adjusted to 6.0 in 0.1 M acetate buffer. The suspension was filled into a column and the settled resin (18 l) was washed with 72 l of water.

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18 l of Mucor miehei lipase (10,100 LU/ml) adjusted to pH 6.0 was recirculated at 30 l/h for 6 hours with pH control. After displacement with 20 l of water a combined volume of 37 l contained 126 LU/ml corresponding to 97% immobilization yield. The column was further washed with another 20 l of water and the preparation was vacuum dried at room temperature whereby 6.0 kg (97% dry matter) immobilized lipase preparation was obtained. The batch interesterification activity was 30.2% POO, 6.9% POP at 1/2 hour or 14.7% P_{inc}.

Application experiment No. 1

4.0 g of the immobilized lipase preparation was filled into a water jacketed column with an internal diameter of 1.5 cm. The temperature in the column was maintained at 60°C. An olive oil/decanoic acid substrate with a composition of 2.5/1 (w/w) was pumped through a precolumn containing 30 g Duolite S 561*saturated with 21 ml of ion exchanged water and further through the main column containing the immobilized lipase preparation. The flow rate was controlled in order to keep the composition of the output at a value corresponding to approximately 65% conversion, i.e. 23% DOO in the final triglyceride (DOO means a triglyceride with one decanoic acid unit (in outer position) and two oleic acid units).

On the assumption that the decrease of the activity of the immobilized lipase follow a first order reaction the half life can be estimated to 3200 hours. With an initial activity of 2.4 g triglyceride/hour/g enzyme preparation the productivity is appromately 8.3 tons of triglyceride /kg enzyme preparation assuming a run time of two half lives. In fig. 1 the logarithm to the flow rate is plotted against the time

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Application experiment No. 2

The same experiment as No. 1 was performed at 70°C instead of 60°C.

The half life was found to be 1300 hours and the initial activity to 2.3 g triglyceride/hour/g enzyme preparation corresponding to a productivity of 3.2 tons of triglyceride/kg enzyme preparation. The logarithm to the flow rate is plotted against time in fig. 2.

Example 14

This example illustrates the potential of an immobilized lipase preparation produced according to the invention for the continuous interesterification of a high-melting triglyceride mixture composed of beef tallow and soy bean oil without solvent or other auxiliary agents. Similar processes may be useful for the preparation of special fats without the use of hydrogenation and chemical interesterification and suitable for margarine or related products.

Immobilization

19.8 grams of moist (86.0% dry matter) Duolite A 561*ion exchange resin, with more than 80% of the particles between 400 and 850 μm, was adjusted to pH 6.0 in aqueous suspension and washed with water. 50 ml of Mucor miehei lipase (7400 LU/ml, 8% dry matter) was mixed with the resin and pH was readjusted to pH 6.0. After stirring for 2 hours at room temperature, filtration and washing with 2 x 50 ml water, the preparation was dried in vacuum at room temperature. The yield was 19.2 grams containing 8.5% water. The activity left in the filtate was 34% of the total, initial amount. Batch interesterification activity was 25.4% POO, 6.0% POO at 1/2 hour or 12.5% inc. P.

Analysis of the interesterification reaction

White beef tallow, and fresh, refined soy bean oil were obtained from local markets. The substrate was composed

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of 1.5 parts of beef tallow and 1 part of soy bean oil which were mixed at 70°C. BHT antioxidant was added in a concentration of 0.1%. To characterize the individual components and to follow the interesterification reaction, HPLC was used to analyze the triglyceride composition of the sustrate components, the initial mixture and the interesterified mixture. An initial batch reaction with 2.75 grams immobilized Mucor miehei lipase preparation, 24 grams tallow, and 16 grams soy bean oil was run 16.5 hours at 65°C. HPLC showed that the ratio of LPO- to LLL-triglyceride (L: Linoleic, P: Palmitic, O: Oleic) in the mixture increased from 0.62 to 1.16, this latter figure presumably being close to the equilibrium ratio.

Melting properties of the interesterified mixture

The change in melting properties due to interesterification was analyzed by dilatation according to the official IUPAC-method (IUPAC: standard methods for the analysis of oil, fats, and derivatives, 6th ed., method No. 2.141 (1979)). The results appear from the table below, with a corresponding non-interesterified mixture of beef tallow and soy bean oil (1.5:1) as a reference.

Temperature, °C		0	20	25	30	35	40	45
Dilatation (µl/g fat)	Non interesterified mixture	30.8	22.9	18.7	14.6	11.2	6.5	1.6
	Interesterified mixture	16.5	4.9	4.9	3.1	0.6		

Test in column

A small thermostatted column system was operated for 2 days to illustrate a continuous process. 4.0 grams of the described immobilized lipase preparation was placed in a column. Also a pre-column containing 5 grams of moist Duolite A 561 resin (50% dry mtter) was used. Beef tallow/soy bean oil in the ratio 1.5:1 w/w was continuously fed through the column system at 67°C. The performance of the immobilized lipase preparation is shown in the table below:

Sample/time	Flow g TG/h/ g enz.	Composition LPO/LLL	Conversion %
Tallow/SBO - substrate (18 h)	-	0.65	~6
18 hour's product	2.10	0.90	52
41 hour's product	1.63	0.93	54
Equil. (batch)	-	1.16	100

Example 15

This example illustrates the application of immobilized lipase according to the invention for fat hydrolysis.

To a 1:1 mixture of pure beef tallow and water, 40 grams of each, was added immobilized lipase, prepared as described in example 13, in an amount corresponding to 100 LU/g fat, or 0.13 grams of immobilized lipase preparation, assuming a load of 30.000 LU/g of immobilized lipase. An effective mixing was obtained by magnetic stirring at 48°C. The initial pH value in the water phase was adjusted to 8.0. After 4 days the enzyme was separated and the fat phase analyzed for degree of hydrolysis (DH). Duplicate tests were carried out. The recovered immobilized enzyme was used for a 2' run and a comparison experiment with soluble lipase, also added in an amount corresponding to 100 LU/g fat, was carried out. In this case the aqueous phase was used for the 2' run. Three tests were carried out with soluble lipase.

The results were as follows:

Preparation	Sample No.	%DH, 4 days, 48°C 1' run	2' run
Immobilized lipase	1	30	31
	2	35	37
Soluble lipase	1	61	5
	2	55	8
	3	46	7

The pH value dropped to about 6.8 with the immobilized lipase and to about 6.3 in the 1' run and to about 7.4 in the 2' run with the soluble lipase. %DH was calculated as the acid value (AV) divided by the saponification value (SV).

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. Method for production of an immobilized lipase preparation intended for interesterification of fats, which comprises contacting an aqueous solution of a microbial lipase derived from Mucor miehei with a particulate, macroporous, weak anion exchange resin which contains primary and/or secondary and/or tertiary amino groups and which has a particle size such that more than 90% of the resin particles have a size between 100 and 1000 μm, at a pH in the range of 5 to 7 to bind said lipase to said resin to form said immobilized lipase, whereby at least 75% of lipase activity is removed from the solution, then separating the immobilized lipase from the aqueous solution and thereafter drying the separated immobilized lipase to a water content of between 2 and 40%.

2. Method according to Claim 1, wherein the lipase is a thermostable lipase.

3. Method according to Claim 1, wherein the microbial lipase is derived from thermophilic Mucor miehei.

4. Method according to Claim 1 wherein more than 90% of the particles of the macroporous weak anion exchange resin has a particle size between 200 and 400 μm.

5. Method according to Claim 1 wherein the proportion between the amount of the aqueous solution of the microbial

lipase and the weight of weak anion exchange resin corresponds to 5,000 - 50,000 LU/g ion exchange resin (dry weight).

6. Method according to Claim 1, wherein the contact time is between 0.5 and 8 hours.

7. Method according to Claim 1, wherein the separation is performed by simple filtration.

8. Method according to Claim 1, wherein the drying is carried out to a water content between 5 and 20%.

9. Method according to Claim 1, wherein the particulate, macroporous, weak anion exchange resin is brought together with an aqueous solution of a crosslinking agent, before, during or after the contact between the particulate, macroporous weak anion exchange resin and the aqueous solution of the microbial lipase, whereafter the remaining solution of crosslinking agent is separated.

10. Method according to Claim 9 wherein said aqueous solution of a crosslinking agent is an aqueous glutaraldehyde solution in a concentration between 0.1 and 1.0% w/w.

11. Method for interesterification of fats, wherein melted fat(s), facultatively mixed with melted free fatty acid, is contacted with the immobilized lipase preparation produced according to any one of Claims 1, 2 or 3.

12. Method for interesterification of fats, wherein melted fat(s), facultatively mixed with melted free fatty acid, is contacted with the immobilized lipase preparation produced according to any one of Claims 4, 5 or 6.

13. Method for interesterification of fats, wherein melted fat(s), facultatively mixed with melted free fatty acid, is contacted with the immobilized lipase preparation produced according to any one of Claims 7, 8 or 9.

14. Method for interesterification of fats, wherein melted fat(s), facultatively mixed with melted free fatty acid, is contacted with the immobilized lipase preparation produced according to Claim 10.

15. Method for hydrolysis of fats, wherein an emulsion of triglyceride and water is contacted with the immobilized lipase preparation produced according to any one of Claims 1, 2 or 3.

16. Method for hydrolysis of fats, wherein an emulsion of triglyceride and water is contacted with the immobilized lipase preparation produced according to any one of Claims 4, 5 or 6.

17. Method for hydrolysis of fats, wherein an emulsion of triglyceride and water is contacted with the immobilized lipase preparation produced according to any one of Claims 7, 8 or 9.

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18. Method for hydrolysis of fats, wherein an emulsion of triglyceride and water is contacted with the immobilized lipase preparation produced according to Claim 10.

19. Method for synthesis of fats, wherein a mixture of glycerol and free fatty acids is contacted with the immobilized lipase preparation produced according to any one of Claims 1, 2 or 3.

20. Method for synthesis of fats, wherein a mixture of glycerol and free fatty acids is contacted with the immobilized lipase preparation produced according to any one of Claims 4, 5 or 6.

21. Method for synthesis of fats, wherein a mixture of glycerol and free fatty acids is contacted with the immobilized lipase preparation produced according to any one of Claims 7, 8 or 9.

22. Method for synthesis of fats, wherein a mixture of glycerol and free fatty acids is contacted with the immobilized lipase preparation produced according Claim 10.

23. An immobilized lipase preparation for interesterification of fats, hydrolysis of fats and/or synthesis of fats which is obtainable by contacting an aqueous solution of a microbial lipase derived from Mucor miehei with a particulate, macroporous, weak anion exchange resin which contains primary

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and/or secondary and/or tertiary amino groups and which has a particle size such that more than 90% of the resin particles have a size between 100 and 1000 μm, at a pH in the range of 5 to 7 to bind said lipase to said resin to form said immobilized lipase, whereby at least 75% of lipase activity is removed from the solution, then separating the immobilized lipase from the aqueous solution and thereafter drying the separated immobilized lipase to a water content of between 2 and 40%.

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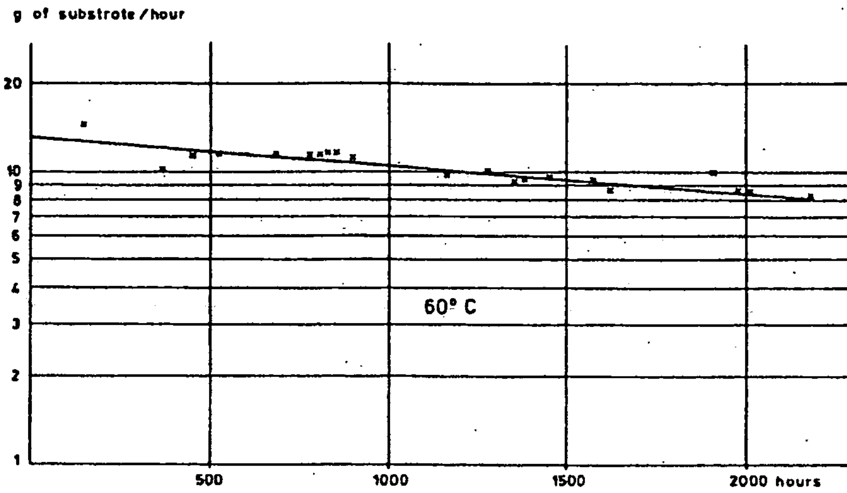

fig.1

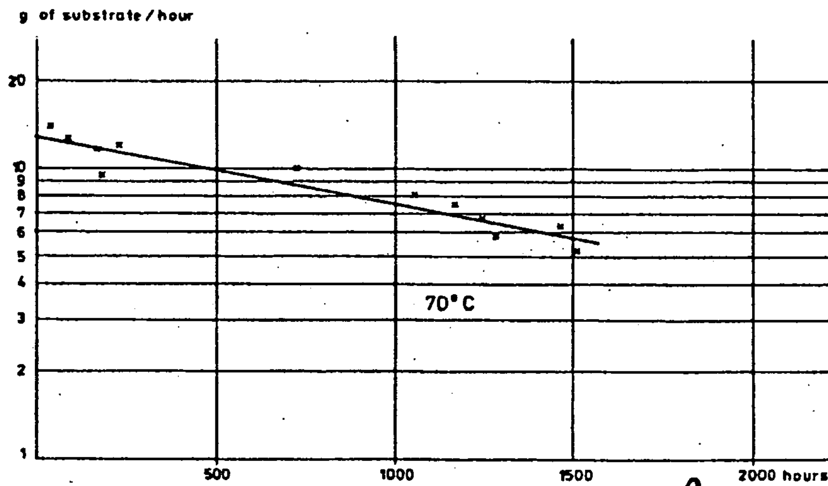

fig.2

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